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Review

Conventional and modern propagation techniques in *Piper nigrum*

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***Piper nigrum,* commonly known as ``Black-pepper``, has gained a global consideration because of its volume in the spice industry. This plant has shown great potential for the discovery of novel biologically active compounds and need for techniques to enhance the production of high quality consistent plant material for feasible accumulation of metabolites. Tissue culture of *P. nigrum* can play a vital role in germplasm conservation, enhanced multiplication and genetic engineering for feasible production of biologically active compounds. Liquid culture is yet to be established and reserves corner for industrial production of these active components. This review provides the developments in the propagation practices and challenges that remain in *P. nigrum* biotechnology.**

Key words: Piper, black-pepper, peppercorn, piperine, tissue culture.

INTRODUCTION

Out of 1000 species of *Piper* (*P.*), *P. nigrum* is the most important cultivated species due to its economic value (Bhat et al., 1995). Geographically, it is confined to Western-Ghats of South India (Nair et al., 2003). However, some reports of cultivation from Malaysia, Indonesia, Brazil, Sri-Lanka and West Indies are also available (Backer et al., 1963). *P. nigrum* had been found in vast altitudinal regions and showed great adaptability to a wide range of environmental conditions which led to inter-species diversity (Howard, 1973). “Black-pepper” as its generalized name is due to the color of the peppercorn. It is considered as the “king of spices” due to its trade in the international market (Srinivasan, 2007; Mathew et al., 2001). Black-pepper is widely used in cooking and processing of food and perfumery (Philip et al., 1992; Bhat et al., 1995). Its quality is judged by its odor and pungency (Kay, 1970). Piperine is an active component in *P. nigrum* and contributes to its pungency (Tripathi et al., 1996).

P. nigrum is reputed in the local system of medicine of India, Latin America and West-Indies for its multi-dimensional medicinal properties (Scott et al., 2008). Secondary metabolites from *P. nigrum* play defensive role against infections by microbes, insects and animals (Lupina and Crrips, 1987). Efforts have been made in screening these chemicals against different pathogenic species of microorganisms (Umit et al., 2009). Piper-amides extracted from *P. nigrum* had shown insecticidal activities (Scott et al., 2005; Boff et al., 2006). β-caryophyllene extracted from *P. nigrum* showed anesthetic activity (Santra et al., 2005). Nerolidol is a very famous secondary metabolite of *P. nigrum*, used to control mites. Another important component of pepper volatile oil is pipene, which is a famous odorants (Jayalekshmy et al., 2003). Black-pepper is anti-microbial (Dorman and Deans, 2000), anti-mutagenic (El-Hamas et al., 2003), a free-radical scavenger (Gulcin, 2005; Saxena et al., 2007), immuno-modulator, anti-tumor (Sunila and Kuttan, 2004), anti-depressant (Lee et al., 2005), anti-apoptotic (Pathak and Khandelwal, 2007), anti-metastatic (Pradeep and Kuttan, 2002), anti-thyroid (Panda and Kar, 2003), hepatoprotective (Koul and Kapil, 1993), immunostimulator (Pathak and Khandelwal, 2009), anti-diarrheal and anti-spasmodic (Bajad et al., 2001). Black-pepper was reported to treat pulmonary diseases (Ravindran, 2000), fever, cold, colic disorder and gastric conditions (Parmar et al., 1997: Kumar et al., 2007). Recently, anti-spermatogenic and infertility effect in mice

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were reported by Mishra and Singh (2009).

FORMAL PROPAGATION PRACTICES

P. nigrum is a perennial climbing vine grown for its berries, the supports (standards) used for establishment of black-pepper may be living or non living, however, the use of non-living standards often resulted in higher yields. A major constraint in the cultivation of crop is its low productivity due to poor genetic stock and incidence of pests and diseases.

Most Indian-cultivars produced seed. Nonetheless, seed production in Black-pepper is uncertain and yields only few heterogeneous progenies due to their short viability and high sterility in post generation stages (Atal and Banga, 1962). Therefore, Black-pepper is conventionally propagated through cutting with 2 - 6 nodes for field plantations. Plantlets have also been obtained from micro-propagated shoot tips of mature vine (Philip et al., 1992) and seedlings (Mathews and Rao, 1984). Plants are traditionally raised from seeds or cuttings; dioecious progenies are usually produced by seeds, however favorable hermaphrodite cultivars can be obtained by vegetative propagation. Few researchers described methods of vegetatively propagating black-pepper for initial multiplication as well as for large-scale planting. Black-pepper is commonly infested by fungal, bacterial, viral and mycoplasmal pathogens. Internal infection caused by viruses and mycoplasma are difficult to control and are transferred by vegetative propagation. Latent pathogens especially viruses often result in the loss of plant production and a poor quality product (Phillip et al., 1992). A number of factors such as high heterozygosity, polyploidy, diocieous nature hamper breeding improvement in *P. nigrum*.

Considerable variation exists among the cultivars with respect to an array of plant morphological characters, both qualitative and quantitative (Mathew et al., 2001). Black-pepper is traditionally propagated by stem cuttings (Nair and Gupta, 2006). Viability of seeds is severely affected by storage (Ravindran et al., 2000; Chaudhury and Chandel, 1994).

IN VITRO TECHNIQUES FOR MASS-PROPAGATION

In vitro techniques offer the possibility of rapid clonal multiplication of elite plant species, allowing production of genetically stable and identical progeny (Hu and Wang, 1983). *In vitro* propagation is an alternative method to traditional propagation (Abbasi et al., 2007; George and Sherrington, 1984). *In vitro* culture offers improvements over traditional vegetative propagation because of faster rate of multiplication. Plant tissue culture has revolutionized the knowledge and application in several fields of the plant kingdom (Cooking, 1986). Tissue culture techniques played an important role in clonal propagation, germplasm conservation and plant improvement of black-pepper (Bhat et al., 1995; Sajc et al., 2000). Combination of *in vitro* propagation and cryopreservation techniques helped in the conservation of black-pepper diversity (Fay, 1992).

In vitro seed germination

The plants are traditionally raised from seeds or cuttings. Seeds are commonly infected by fungal, bacterial, viral and mycoplasmal pathogens (Philip et al., 1992). Endogenous bacterial-infestation caused severe setback to *in vitro* establishment of *P. nigrum* (Mathews and Rao, 1984; Fitchet, 1990; Philip et al., 1992). Surface sterilization of seeds by ethanol, sodium hypo-chloride and mercury chloride (Mujib, 2005; Azad et al., 2003) was found to be effective. Repeated surface sterilization has been reported to delay the onset of bacterial growth but could not eliminate it (Fitchet, 1990; Philip et al., 1992).

Explants

Choice of explant plays an important role in determining the efficiency of propagation (Abbasi et al., 2007). Morphogenetic potential of root, leaf, node and internode explant of *P. nigrum* was investigated (Bhat et al., 1995). Successful *in vitro* techniques for micropropagation of black-pepper have been reported using mature shoot-tip explants (Nazeem et al., 1992; Philip et al., 1992; Babu et al., 1993; Joseph et al., 1996; Philip et al., 1992). Leaf explant (Sujatha et al., 2003), nodes explant (Bhat et al., 1992), seeds (Nair and Gupta, 2006) have been exploited as explant material. Plantlets regenerated from seedling-derived callus and shoot apices had been reported, but most attempts to regenerate plants from mature vine were unsuccessful (Mathews and Rao, 1984). The source of explant was important in determining the morphogenetic and regenerative potential, which were significantly influenced by the physiological conditions of the donor plant (Debergh and Maene, 1981; Read, 1988). Maintaining the donor plants in clean and controlled environmental conditions delivers healthy and sterile explants (Sagare et al., 2001). The physiological age of explants and the explant type and size are the other factors which influenced formation of organs *in vitro* (Rout et al., 2000).

Regeneration

In vitro regeneration system had been exploited for this vegetatively propagated species. Both organogenesis and somatic embryogenesis were successfully induced

on solid and liquid basal medium (Schenk and Hildebrandt, 1972; Murashige and Skoog, 1962).

Shoot organogenesis

Organogenesis refers to the initial and subsequent *de novo* growth of organs and plantlets directly or indirectly (Nalawade and Tsay, 2004). Plant growth regulators concentrations in the medium and additional media amendments also play a determining role in morphogenesis (Narayanaswamy, 1977). Leaf explants cultured on modified MS medium supplemented with Indole acetic acid (IAA) and Benzyl aminopurine (BAP) 1 µM produced creamy white to pale green friable calli. Addition of silver nitrate from 5 – 15 µM increases shoot-regeneration frequency. BAP alone or in combination with Indole butyric acid (IBA) and Adenosine sulphate ($AdSO_4$) support initial proliferation of shoot tip explants, BAP more than 5 µM suppress growth of shoots and proliferation , BAP 1.5 µM and IBA 3 µM produced optimized results (Philip et al., 1992). At higher concentrations of BAP (5 - 10 µM), only 40% of the nodal explants of *P. nigrum* responded. Kinetin (Kn) failed to induce shoot buds in *P. nigrum* but supported axillary bud proliferation (Bhat et al., 1995). Combination of BAP and activated charcoal had a significant effect on the number of shoots. Shoot tip and nodal explants failed to stimulate bud break and shoot formation on cytokinin-free medium. Increasing the concentration of BAP (0-6.6 µM) enhanced the growth response in both type of explants. Higher concentrations of BAP and kinetin suppressed number of shoot formation. Up to 88% response was observed for 4.43 µM BAP and 2.32 µM Kn (Anand and Rao, 2000). Best shoot organogenesis was achieved by the interaction of Kn and IAA, while BAP and IAA combinations proved to be considerably ineffective (Rubluo and Barroso 1992). Gunay and Rao (1978) concluded that Kn was inefficient in inducing differentiation and reported only callus production.

Somatic embryogenesis

Somatic embryogenesis refers to the formation of an embryo from a cell other than a gamete or a direct product of gamete fusion. Somatic embryos develop directly into differentiated plant parts through differentiation (Dudits et al., 1995). In cyclic somatic embryogenesis, unlimited numbers of embryos are proliferated in a repetitive manner from single culture of primary embryos (Singh and Chaturvedi, 2009; Raemakers et al., 1995). High frequency plant regeneration through somatic embryogenesis is a suitable system for mass propagation of plants (Nair and Gupta, 2006). Plant regeneration through direct somatic embryogenesis from the micropylar tissues of the germinating seeds of black-pepper and its scaling up through high-frequency cyclic secondary somatic embryogenesis have been described in earlier reports (Nair and Gupta, 2003). High frequency secondary somatic embryo proliferation was achieved subsequently on plant growth regulator-free SH medium containing 1.5% sucrose (Nair and Gupta, 2006). Embryogenic suspension cultures were established using the prolife-rating secondary embryogenic clumps as inoculum and uniformity of cultures was induced by sieving through a mesh of 500 µM (Nair and Gupta, 2006).

Secondary embryos formed from the radicular end of the primary somatic embryos which were originally derived from micropylar tissues of germinating seeds on PGR-free SH medium in the absence of light. The process of secondary embryogenesis is carried out in a cyclic manner from the root pole of newly formed embryos resulting in clumps of somatic embryos (Nair and Gupta, 2005). A single-flask system was standardized for proliferation, maturation, germination and conservation of secondary somatic embryos in suspension cultures. The system of cyclic secondary somatic embryogenesis in black-pepper described here represents a permanent source of embryogenic materials that can be used for genetic manipulations of this crop species (Nair and Gupta, 2005). It can also be used in species where zygotic embryos contain important secondary metabolites (Raemakers et al., 1995). Somatic embryogenesis also overcomes post fertilization barriers of the embryo, immature embryos of inter-specific plants from incompatible crosses may be rescued by culturing them for somatic embryogenesis and simultaneously the plant can be multiplied.

Root organogenesis

The process of *in vitro* root initiation, development and elongation normally require medium containing auxin (Azad et al., 2003). For better rooting, shoots were excised from *in vitro* plant 4.5 cm in length and transferred to ½ strength MS basal medium containing 1 µM Naphthalene acetic acid (NAA). Each shoot produced 8-10 roots, the culture derived plant resembled normal field grown plants (Philip et al., 1992). All shoots regenerated 2-4 roots within 2 weeks of transfer to a medium containing 1 µM IAA; such regenerated plants were successfully established in soil (Bhat et al., 1995; Azad et al., 2003). For other *Piper spp.* the micro shoots obtained through *in vitro* shoot multiplication and adventitious shoot regeneration were rooted in ½ and ¼ MS basal medium under light (16/8 h photoperiod) and dark culture conditions. 100% rooting response was observed under dark incubation.

Acclimatization of regenerated plantlets

Four week old *in vitro* regenerated plants were washed in tap water and transplanted into pots filled with sterilized ``soil rite``. Pots were covered with polythene bags to

provide high humidity around plants and kept in shade in a net house. Polyethene covers were gradually removed over a period of 2 weeks and after 4 weeks plants were transferred to soil (Bhat et al., 1995). 4-5 cm long shoots were rooted on ½ strength MS medium containing 1 µM NAA after 4 weeks. Rooted plantlets were removed from the medium and transferred to pots containing compost (peat, 2: sand, 1: soil, 1) and covered with transparent polythene for two weeks before transferred to the greenhouse (Philip et al., 1992). The prevailing conditions (humidity and temperature) of transplanting season greatly influenced the initial survival of potted plantlets (Azad et al., 2003). The plantlets were transplanted to soil and acclimatized in the growth chamber under high humidity conditions. The rooted plants were transferred to field nursery for hardening (Anand and Rao, 2000).

GENETIC ENGINEERING

There has been a limited research on genetic engineering of *P. nigrum*. Recently a very efficient micropropagation strategy through somatic embryogenesis was reported (Nair and Gupta, 2006), which enabled genetic manipulations of this crop for specific traits including abiotic and biotic stress tolerance. Often, improvement of transgenic traits in plants is achieved by using specific promoters to accomplish high tissue-specific protein production in transgenic plants (Abbasi et al., 2007). In *P. nigrum*, promoters with root and leaf targeted expression ensured that the gene product was not present in the edible portions. Native promoters were known to mimic the expression of endogenous genes more effectively and did not appear to be greatly affected by gene silencing (Song et al., 2000). Suppression subtractive hybridization (SSH) was used to successfully generate a defense gene enriched subtracted cDNA library in the resistant wild pepper. SSH was used to identify a set of genes differentially expressed in the leaves of *P. nigrum*, which could facilitate targeted engineering of valuable crop. A PCR-based SSH technique was used to generate a leaf-specific subtracted cDNA library of *P. nigrum*. A tester population of leaf cDNA was subtracted with a root derived driver cDNA. The efficiency of subtraction was confirmed by PCR analysis using the housekeeping gene actin. On sequence analysis, almost 30% of clones showed homology to metallothionein type-2 gene. The predominance of metallothionein transcripts in the leaf was further confirmed using Real-time PCR analysis and Northern blotting. The possible role of metallothionein type-2 homologues in the leaf is discussed along with the feasibility of using SSH techniques for identification of more number of tissue-specific genes from *P. nigrum* (Alex et al., 2008). SSH techniques have been efficiently used for identification of many differentially expressed plant genes (Chou et al., 2006) and for generation of equalized and enriched tissue-specific cDNA library (Stahl et al., 2004). One of the advantages of using SSH is the normalization step involved, which is highly efficient in removing the transcripts common to driver and tester population, allowing the enriched expressed cDNA in the subtracted population. Typically, plant metallothioneins are Cys-rich proteins and further categories as type 1 and 2 have been designated on the basis of predicted location of Cys residues (Scott and Albert, 2005). Metallothioneins type-2 transcripts have been detected primarily in mature leaves. The high levels of expression of metallothioneins type-2 in leaves, especially in trichomes is correlated to the secretory function of trichomes to exude excess heavy metals accumulating preferentially in leaves (Garcia-Hernandez et al., 1998) and leaf trichomes produce secretions that are thought to provide a first line of defense against pests and pathogens (Wang et al., 2002). A comparative study on accumulation of Cd, Fe, Cu, Mn and Zn in commonly used tropical spices indicated a highest concentration of these metals in the leaves and seeds of *P. nigrum* (Ozkuthu et al., 2006).

PHYTOCHEMISTRY

The following biologically important phytochemicals have been extracted from *P. nigrum* plants: alkaloids, amides, propenyphenols, lignans, neolignans, terpenes, steroid, kawapyrones, piperolides, chalcones, dihydrochalcones, brachyamide (Kiuchi et al., 1988), dihydropipericide (Miyakado et al., 1980), 3,4-dihydroxy-6(N-ethyamine), benzamide (Bandyopadhyay et al., 1990), (2E , 4E)-N-eicosadienoyl pereridine (Kiuchi et al., 1988), N-trans-feruloyltryamine, N-formylpiperidine (Stohr et al., 2001), guineensine (Kiuchi et al., 1988), (2E , 4E)-N-5[(4-Hydroxyphenyle)-pentadienoyl] piperidine, (2E,4E)-N-isobutyldecadienamide), (2E,4E)-N-isobutyl-eicosadienamide (Bano et al., 1991), (2E,4E,8Z)-N-isobutyl-eicosatrienamide, (2E,4E)-N-isobutyloctadienamide (Nakatani et al., 1980), piperamide, piperamine (Kiuchi et al., 1988), piperettine (Orjala et al., 2003), pipericide (Miyakado et al., 1980), piperine, piperolein, trichostachine, sarmentine, sarmentosine, tricholein, retrofractamide (Kiuchi et al., 1988). Concentration of alkaloids in fruits of *P. nigrum* ranges from 4 to 5% (Dev and Koul, 1997). Pino et al., 2003 observed that the major components of the essential oil obtained from the aerial parts of *P. nigrum* were gluulol, α-pinene, β- caryophyllene and α-terpinene. Piperine was the first amide to be isolated from piper species. Piperine the major active principle of black pepper, is closely related in structure to the known natural carcinogens-safrole, estragole and methylengenol which are also widely distributed in spices and plant oils (Ames, 1983).

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